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(71) Applicant: THE REGENTS OF THE UNIVERSITY OF COLORADO [US/US]; 914 Broadway, Campus Box 51, Boulder, CO 80301 (US).

(72) Inventors: SHEFTER, Eli ; 17251 Knoll Trail, #1501, Dallas, TX 75248 (US). MANNING, Mark, G. ; 1112 Live Oak Court, Fort Collins, CO 80525 (US).

(74) Agents: GREGG-EMERY, Valeta, A. et al.; Beaton & Swanson, 4582 S. Ulster Street Parkway, Suite 403, Denver, CO 80237 (US).

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(57) Abstract

A hydrophobic ion-pairing (HIP) complex is formed by the addition of an anionic surfactant, such as sodium dodecyl sulfate (SDS), to a polypeptide, protein, or other molecule in solution. Formation of an HIP complex alters the solubility and partitioning behavior of molecules. The HIP complex forms a precipitate which can be redissolved in an organic solvent to form a true homogeneous solution. The HIP complex partitions into an organic solvent at a rate of 2-4 orders of magnitude greater than that for uncomplexed material. The resulting protein maintains its structural and biological activity and exhibits a large increase in thermal stability. The HIP complex can be subsequently extracted back into an aqueous medium with retention of the native protein structure. Enzymes dissolved as an HIP complex can perform catalytic transformations in nonaqueous media. The homogeneous solution formed by the HIP complex in organic medium is useful in the formulation of a variety of systems to administer the drug to the body. The HIP complexation process can be controlled to yield in particles of a desired specific size. For example, for pulmonary delivery, proteins can be formulated into particles of 2-10 microns, within the particle size required for effective pulmonary delivery.

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ION-PAIRING OF DRUGS FOR IMPROVED EFFICACY AND DELIVERY

FIELD OF THE INVENTION

This invention relates to a method of modulating the solubility and partitioning behavior of polypeptides, proteins and other molecules through the use of ion pairing agents. A hydrophobic ion-pairing (HIP) complex is formed by the addition of an anionic surfactant, such as sodium dodecyl sulfate (SDS), to a protein in solution. The HIP complex forms a precipitate which can be redissolved in an organic solvent to form a true homogeneous solution. The resulting protein maintains its structural and biological activity and exhibits a large increase in thermal stability. The uncomplexed protein can be subsequently extracted back into an aqueous medium with retention of the native protein structure. Enzymes dissolved as an HIP complex can perform catalytic transformations in nonaqueous media. The homogeneous solution formed by the HIP complex in organic medium is useful in the formulation of a variety of systems to administer drugs to the body.

BACKGROUND OF THE INVENTION

It has been known for some time that anionic detergents, such as sodium dodecyl sulfate (SDS), can interact in a specific fashion with proteins and polypeptides (Oakes and Cafe (1973) Eur. J. Biochem. 36:559-563; Satake and Yang (1973) Biochem. Biophys. Res. Commun. 54:930-936; Braude and De Clerq (1979) J. Biol. Chem. 254:7758-7764). At low concentrations of SDS, the aqueous solubility of a polypeptide can be severely diminished (Satake and Yang (1973) supra). At higher concentrations, aqueous solubility is regained, presumably via micellar solubilization. While it has not been proven conclusively, it appears that the SDS molecules displace common counter ions, such as chloride, from the ion pairs on the protein.

Replacement of the hydrophilic counter ions by the more hydrophobic SDS molecules alters the solubility properties of the protein.

The interaction between anionic detergents containing long chain alkyl groups, such as SDS, and polypeptides is termed hydrophobic ion-pairing (HIP). Substances modified in this fashion are termed HIP derivatives. These interactions should not be confused with the behavior of proteins in the presence of high concentrations of SDS, as used in gel electrophoresis (Pitt-Rivers and Impiombato (1968) Biochem. J. 109:825-830; Steinhardt et al. (1974) Biochemistry 13:4461-4468; Steinhardt et al. (1977) Biochemistry 16:718-725). SDS is not the only compound which can modify solubility properties in this way. Any hydrophobic material that is the salt of an acid can be employed, including sulfates, sulfonates, phosphates, and carboxylates. As sulfates are the salts of the stronger acids in the series, they should be the most efficient at forming ion pairs. In fact, it has been observed that for interaction with mouse interferon, only sulfates and not sulfonates or carboxylates form strong ion pairs (Braude and De Clerq (1979) supra). Conversely, there has been a preliminary report that leuprolide, a nonapeptide, forms ion pairs with alkyl sulfonates (Adjei et al. (1989) Pharm. Res. 7:565-569). Neither the Braude and De Clerq (1979) supra or Adjei et al. (1989) studies involved formation of an HIP complex precipitate.

The formation of ion-pairs between a surfactant and drugs has been investigated as a method of improving drug delivery and membrane permeability (Tomlinson and Davis (1980) J. Colloid. Interf. Sci. 74:349). It is generally accepted that drugs in their non-ionized form have better transport properties through biological membranes compared to their ionized forms (Cools and Jansen (1983) J. Pharm. Pharmacol.

35:689-691). Peptides with multiple sites for ionization at physiological pH present a major problem from the standpoint of drug delivery since charged molecules have a low lipid solubility (Hirai et al. (1981) Int. J. Pharm. 7:317-325). One way to overcome the adverse effect of ionization is to use a lipophilic counterion to form an ion-pair and facilitate partitioning into the biological membrane (Okada et at. (1983) J. Pharm. Sci. 72:75-78). For example, Mazzenga and Berner (1991) J. Controlled Release 16:77-88, describe an improved method for transdermal delivery of zwitterionic drugs. Their objective was to maximize the solubility of zwitterions in both polar and nonpolar media. Their method is limited to selecting a solvent in which the zwitterion salts are soluble and which also swells the skin or membrane, a solvent characteristic necessary for transdermal delivery of zwitterions. Hydrohalide, alkyl sulfonate, maleate, and fumarate salts of the zwitterions phenylalanine, baclofen, and two diacid angiotensin converting enzymes were examined for solubility in water, ethanol, octanol, and chloroform. Mazzenga and Berner found that within the alkyl sulfonate series, the ethane sulfonate salt showed an optimal 2-fold increase in solubility, and further increases in chain length had minimal additional effects. Although Mazzenga and Berner (1991) supra tested HIP complex precipitates for solubility, their results teach away from the use of longer chain alkyl groups as a means of increasing protein solubility in organic solvents.

Efforts directed toward the improvement of drug absorption have focused on substances that facilitate the transport of solutes across biological membranes, termed penetration enhancers (Lee et al. (1991) Critical Rev. Therap. Drug Carrier Systems 8:91-192). Nishimura et al. (1985) Chem. pharm. Bull. 33:282-291 and Van Hoogdalem et al. (1988) Pharm. Res. 5:453-456,

reported enhanced rectal absorption of drugs with sodium caprate (C_{10}). Mishima et al. (1987) J. Pharmacobiodyn. 10:624-631, reported that sodium caprate increased the nasal absorption of insulin. Corneal penetration of a hydrophilic compound and a macromolecular compound has been shown to be enhanced by addition of sodium caprate (Morimoto et al. (1989) Arch. Int. Pharmacodyn. 302:18-26). SDS administered at a concentration of 1% has been shown to enhance nasal absorption of insulin (Lee et al. (1991) supra). Other surfactants have also been shown to promote percutaneous transport of naloxone (Aungst et al. (1986) Int. J. Pharm. 33:225-234). The overall effect of these additives or penetration enhancers is to increase the lipophilicity (hydrophobicity) of the drug in order to increase its ability to partition across a biological membrane. In all of these studies, increases in drug availability were achieved with the use of surfactant concentrations above the critical micelle concentration (CMC). Above the CMC, micelles resolubilize proteins in the water phase. Further, the concentrations of surfactants used in these studies had a direct influence on the barrier membrane. The structure of this membrane was sufficiently altered to allow the drugs to penetrate. By contrast, the present invention represents a completely different approach to the enhancement of drug availability through the use of surfactant concentrations below the CMC.

Implantable systems have been investigated as a means for sustained and controlled drug delivery. One type of drug delivery system is the passive reservoir or matrix type devices in which the drug diffuses through or across a rate limiting barrier (such as a membrane). A central problem concerning membrane-type delivery systems is the permeability of the membrane, and the means to trigger or regulate it by means of some external agency without serious damage to the

living tissue in which the devise is implanted. Another type of matrix system involves biodegradable polymers in which the drug contained therein is released as the matrix is dissolved. Unfortunately, most biodegradable polymers are not soluble in water, whereas almost all proteins and basic drugs are insoluble in organic solvents.

A common method of obtaining a sustained-release product is to embed or disperse the solid drug in an insoluble matrix by compression of a physical mixture of the drug with a polymeric material (see, for example, Wang (1991) Biomaterials 12:57-62). Studies have shown that the addition of a surfactant into the compressed physical mixture increased drug release from such matrixes (Daly et al. (1984) Int. J. Pharmceutics 18:201-205). Feely and Davis (1988) Int. J. Pharm. 41:83, observed a linear decrease in the release of chlorpheniramine maleate (CAM) with increasing concentration of SDS, which appeared to be due to formation of a poorly soluble complex between CAM and SDS. Precipitation titration of solutions of procaine hydrochloride with solutions of SDS produced the same result (Feely and Davis (1988) supra), apparently due to formation of a 1:1 complex between the anionic SDS and the oppositely charged procaine. Complex formation reduced the solubility of procaine hydrochloride in the solution within the pores of the matrix and slowed its release (Wells and Parrott (1992) J. Pharm. Sci. 81:453-457. Although these precipitation titration studies reveal the use of SDS to form HIP complex precipitates, the solubility of the precipitate in organic solvents was not investigated nor suggested.

In order to incorporate water soluble materials, such as peptides and proteins, into biodegradable polymers, emulsion or suspension techniques must be employed, as it is rare that both components will be soluble in the same solvent. Consequently, much of the

peptide may be dispersed near or on the surface, leading to the so-called "burst effect", in which, a significant amount of the drug is released within the first hours of administration. After that point, the system exhibits controlled release of the substance. Therefore, numerous studies have examined emulsion methods for increasing dispersion of the peptide through the polymer, thereby minimizing the burst effect. However, if the polypeptide could be dissolved in a polymer solution of the biodegradable carrier, homogeneous incorporation could be achieved and the burst effect eliminated.

Lipid vesicles have been shown to increase the intestinal absorption or oral bioavailability of water-soluble drugs. Triglyceride oils increased cefoxtin absorption in rats (Palin et al. (1986) Int. J. Pharmaceut. 33:99-104), sodium laurate increased the intestinal absorption of fosfomycin in rats (Ishizawa et al. (1987) J. Pharm. Pharmacol. 39:892-895), and entrapment of carboxyfluorescein in oleic acid vesicles increased its intestinal absorption in rats (Murakami et al. (1986) Pharm. Res. 3:35-40). In these examples, the lipid vehicles may have altered the physical-chemical properties of the cellular membrane, thereby increasing intestinal membrane permeability (Muranishi (1985) Pharm. Res. 2:108-118; Van Hoogdalem et al. (1989) Pharmac. Ther. 44:407-443).

Researchers have investigated the enzymatic ability of proteins to function in nonaqueous media (Matiasson and Aldercreutz (1991) Trends Biotechnol. 9:394-398; Gupta (1992) Eur. J. Biochem. 203:25-32). Klibanov and co-workers have shown that proteins can retain activity when suspended in organic solvents, such as octane (Klibanov (1986) CHEMTECH 16:471-489, and (1990) Acc. Chem. Res. 23:114-120; Zaks and Klibanov (1988a) J. Biol. Chem. 263:3194-3201, and (1988b) J. Biol. Chem. 263:8017-8021; Russell and

Klibanov (1988) J. Biol. Chem. 263:11624-11626). Other approaches that can be used to distribute enzymatic proteins in organic solvents, include reverse micelles (Martinek et al. (1987) Biocatalysis 1:9-15), microemulsions (Luisi and Laane (1986) Trends Biotechnol. 4:153-161), and attachment to polymer supports (Matiasson and Aldercreutz (1991) supra; Gupta (1992) supra). Phase transfer systems which can support enzymatic activity are also known (Semenov et al. (1987) Biocatalysis 1:3-8; Brink et al. (1988) Enzyme Microb. Technol. 10:736-743). In addition, chemical modification, such as the attachment of polyethylene glycol (pegylation) has been used to alter the solubility properties of enzymes (Inada et al. (1988) Trends Biotechnol. 6:131-134). All of these approaches for solubilization of polypeptides and proteins in organic solvents are fundamentally different from that of the present invention. The prior art approaches either use direct suspension of lyophilized protein in nonpolar (alkane) solvents, or the use of reverse micelles. When detergents are involved in the formation of reverse micelles, the concentration of detergent used is well above the CMC.

BRIEF SUMMARY OF THE INVENTION

The present invention involves a method for forming a homogeneous solution of an organic molecule with improved thermal stability over the native molecule comprising adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate from an aqueous solution, isolating the precipitate, and dissolving the precipitate in an organic solvent. In a preferred embodiment of the present invention, the anionic detergent used to form a precipitate is sodium dodecyl sulfate (SDS). In another preferred embodiment of the present invention,

the precipitate formed is dissolved in an organic solvent, such as octanol.

One embodiment of the present invention encompasses a method of forming an injectable implant comprising adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate, isolating the precipitate formed, and co-dissolving the precipitate and a hydrophobic polymer in an organic solvent to form a homogeneous distribution of the organic solvent in the polymer.

Included in this invention is a method for controlling the size of particles of the HIP complex comprising controlling the rates of the mixing of a protein solution and the addition of an anionic detergent to the protein solution. In one embodiment of the method of the present invention, a protein suitable for pulmonary delivery is produced by rapidly mixing a protein solution, adding an anionic detergent dropwise to the rapidly mixing protein solution such that the protein forms HIP complex particles of between 2-10 microns, isolating the particles from solution, and suspending the particles in a suitable pharmaceutical carrier.

Included in this invention is a method of enhancing the thermal stability of proteins comprising adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate, isolating the precipitate, and dissolving the precipitate in an organic solvent.

Included in this invention is a method of incorporating proteins into lipid vesicles comprising adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate, isolating the precipitate, and dissolving the precipitate in a lipid.

In one embodiment of the method of the present invention, the bitter taste of drugs is reduced by

administering a bitter tasting drug in an HIP complex comprising adding a sufficient amount of anionic detergent to a bitter-tasting drug in solution wherein an insoluble precipitate is formed, isolating the precipitate, dissolving the precipitate in an oil, mixing the oil in an aqueous solution to form an emulsion, and administering the emulsion to a patient in need of the drug.

Proteins which may be used in the present invention include, but are not limited to, interleukins, interferons, growth factors such as mammalian growth hormone, basic fibroblast growth factor, transforming growth factors, insulin-like growth factors, calcitonin gene-related peptide, amylin, insulin, somatostatin, octroetide, vassopressin, and colony stimulating factors.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the log of the apparent partition coefficient for the dipeptide Gly-Phe-NH_2.

Figure 2 shows the log of the apparent partition coefficient for 8-Arg-vasopressin (AVP).

Figure 3 shows the log of the apparent partition coefficient for insulin.

Figure 4 shows the CD spectra of a 6:1 SDS-insulin complex in 1-octanol.

Figure 5 shows the CD spectra of insulin extracted from 1-octanol using an aqueous solution of 0.10 M HCl.

Figure 6 shows the effect of temperature on the denaturation of insulin dissolved in 1-octanol.

Figure 7 shows the logarithm of the apparent partition coefficient of bovine pancreatic trypsin inhibitor (BPTI) from pH 4 water into 1-octanol.

Figure 8 shows the UV-visible absorption spectrum of human serum albumin (HSA) in NMP (50:1 SDS to HSA ratio). Figure 9 shows the melting point of the SDS:insulin HIP complex as a function of the molar

ratio of SDS to insulin.

Figure 10 shows a CD scan for a 9:1 SDS:insulin molar ratio at 222 nm as a function of temperature.

Figure 11 shows an absorbance scan for a 9:1 SDS:insulin molar ratio at 222 nm as a function of temperature.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes important advances in HIP technology. The present invention involves the use of ion pairing agents to modulate the solubility and partitioning behavior of polypeptides, proteins and basic drugs. These materials are formed by stoichiometric interaction of a detergent (e.g., alkyl sulfate, such as sodium dodecyl sulfate (SDS)), with the basic functional groups of a polypeptide, protein, or organic molecule that are accessible for ion pairing. The basic group may be an amine (as found in the lysine amino acid residue or the N-terminal amino group of a polypeptide) or a guanidinium group (as in arginine). An ion pair is subsequently formed, referred to as a hydrophobic ion pair (HIP) complex. The complex formed will have reduced aqueous solubility, but enhanced solubility in organic solvents.

The present invention is premised on the discovery that an HIP complex precipitate may be dissolved in an organic solvent to form a true homogeneous solution. Included in the present invention is the discovery that the native tertiary structure of the protein is retained even when dissolved in organic solvents such as 1-octanol. The method of the present invention for forming a true homogeneous protein solution is fundamentally different from any other method for placing proteins into organic solvents, such as those which use suspensions, micelles, microemulsions, or chemical modifications of the protein. This discovery

holds important implications in the area of drug delivery and release, including delivery to the body by inhalation and dispersion in a hydrophobic biodegradable matrix. While the decreased aqueous solubility of the HIP complex has been observed previously, the use of the HIP complex precipitate for improved drug delivery is novel. Measurement of the apparent partition coefficient, defined as the ratio of the equilibrium concentration in an organic phase to that in an aqueous phase, demonstrates that the solubility of a protein in an HIP complex in the organic phase is greater by 2-4 orders of magnitude relative to the chloride salt of the peptide or protein.

Included in the present invention is the discovery that the precipitation of the HIP complex out of aqueous solution may be controlled for the production of uniform HIP complex particles of a desired size. These particles may then form a suspension of the protein. This invention also includes a method of obtaining protein HIP complex particles of specific sizes by controlling the conditions of HIP complex precipitation.

The discovery that HIP complex precipitation can be controlled so as to yield particles of specific size can be exploited to effect the rate of drug released from suspensions. In one embodiment of a method of the present invention, the size of HIP complexes is controlled by controlling the rates of the mixing of a protein solution and the addition of an anionic detergent to the protein solution. The HIP complex can produce very fine suspensions which have limited solubility in water, and the technology can be used to produce particles of varying specific size. The particle size of the HIP complex which is formed in water will depend on the degree of agitation of the protein solution and the rate of counterion addition.

The smallest particles are produced with high shear being applied to the aqueous protein solution and slow addition of SDS. This approach is also important in pulmonary drug delivery, where the particle size is critical to delivery to certain sites within the lung. To obtain particles which will be capable of depositing in the pulmonary region upon inhalation, a high speed homogenizer can be used to stir the protein solution and SDS is added dropwise to the agitated solution. Particles in the 2-10 micron range can be obtained using this procedure. Particles of this size are required to get a sufficient amount of protein delivered to the lung to have a beneficial effect. The particles once formed can be separated by centrifugation or filtration. Larger particles will be formed with slow agitation speeds and more rapid addition of SDS. One example of a protein which could benefit from formation into a fine suspension of HIP complex is DNase, an enzyme currently being used by cystic fibrosis patients to dissolve viscous fluid build up in the lung. Other examples include protein and peptide enzyme inhibitors currently being tested for the treatment of emphysema.

The present invention includes a method of controlling the release of a protein from a suspension by controlling the size of the HIP complex particle. The release rate of protein into an aqueous solution from an HIP complex will be much slower than that of the protein itself. This rate will be a function of the particle size of the complex and the solubility of the complex in water or biological fluid. The solubility is a function of detergent or alkyl acid used and the strength of its association with the protein. Therefore, extended (controlled) release of the protein from the suspension can be achieved. This property permits proteins to be formulated as a suspension for depot injection.

This invention also includes the discovery that uncomplexed protein released from the HIP complex can be extracted back into aqueous medium with retention of its native structure. The native uncomplexed protein can be reclaimed by dissolution in an aqueous solution which contains an excess of chloride or other counter ion, indicating that the complexation is an entirely reversible process. The inventors have discovered that the protein of the HIP complex subsequently extracted back into an aqueous medium retains its native structure. This makes HIP methodology useful in the delivery of proteins for use as therapeutic agents.

An important and unique aspect of the present invention is the discovery that HIP complexes display greatly enhanced thermal stability relative to the native protein, both with respect to chemical degradation and denaturation. This suggests that the HIP complex is useful for long term storage of the protein. Further, this aspect of the invention permits high temperature (steam) sterilization of proteins without the loss of biological activity, which until now, could not be accomplished. Currently, polymer delivery systems for proteins are usually sterilized by radiation as proteins are destroyed by heat. The present invention discloses a method by which proteins may be processed by heating at sterilizing temperatures. Further, the enhanced thermal stability of the present invention may be important for the formulation of proteins in maintaining an active enzyme in an organic solvent, and for long term storage of sensitive proteins.

Included in this invention is a method of uniformly distributing a drug throughout a hydrophobic polymer comprising adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate, isolating the precipitate, and co-dissolving the precipitate and a hydrophobic polymer in

an organic solvent to form a homogeneous distribution of the organic molecule within the polymer.

Many of the current systems for the controlled release of proteins make use of biodegradable polymers. There are at least two major problems with such systems. Under the prior art, a protein can only be suspended during the incorporation process, and because of its polar surface does not suspend well. The term "suspension" refers to the dispersion of a substance or substances in another where the boundaries between them are well defined. A material is dispersed in a solvent where the material has limited solubility in that solvent. This leads to an uneven distribution of the drug and irreproducible drug release profiles. Secondly, the water-soluble drug is leached out of the polymer by biological fluids (rather than its controlled release as the polymer is slowly degraded).

The present invention provides a new method for distributing a drug uniformly through a hydrophobic polymer. HIP complex formation permits both proteins and hydrophobic polymers to possess similar solubility parameters, thus facilitating incorporation of the protein into the polymer matrix. The present inventors have discovered that HIP complexes may be uniformly distributed in biodegradable polymers as they possess a solubility in solvents that will also dissolve the polymer. Where the HIP complex does not dissolve in the solvent used it will suspend easily as a result of its hydrophobic surface.

The present invention wherein the drugs being delivered are included in the polymer matrix in an HIP complex represents three advantages over the biodegradable polymer systems: (1) the hydrophobic polymers can be better mixed with the drug in its lipophilic ion-pair state; (2) the drug forms hydrophobic particles within the polymer, and avoids the problem of the formation of a concentration of

polar particles at the interface of the polymer leading to the "burst" effect; (3) the hydrophobic particles dispersed within the biodegradable polymer are not leached out by biological fluids which result in a predictable release rate. The inventors have discovered the use of the HIP complex to control (retard or extend) the release of a drug at a predictable rate, resulting in part from a more uniform formulation.

One embodiment of this invention includes a method for achieving a true homogeneous solution of biologically active proteins and polypeptides in a organic solvent. None of the methods by which enzymatic activity is achieved in a non-aqueous environment achieve a true protein solution. The present inventors have discovered that the HIP complex can be redissolved in an organic solvent such that a true homogeneous solution is formed. This discovery has important ramifications for controlling the enzymatic activity of proteins in the body. Through the formation of HIP complexes, enzymes and other proteins can be solubilized in a variety of organic solvents, including ethanol, propylene glycol and glycols in general, and N-methyl pyroladone (NMP). These materials should have altered enzymatic activity and specificity. It is important to note that use of HIP to form true solutions of biologically active proteins and polypeptides is a fundamentally different approach from any previously described for achieving enzymatic activity in nonaqueous media.

Also included in this invention is the discovery that the HIP complex dissolved in organic solvent can be extracted back into aqueous medium with retention of the native protein structure. This discovery has potential use in the purification of proteins. A protein having a pI different from others in a mixture may be extracted or preferentially precipitated from

the mixture via HIP complex formation.

The present invention further includes a method of obtaining a stabilized protein comprising precipitating a protein in the HIP complex. Much research effort has been directed into developing stabilized lyophilized formulations of proteins, including by the addition of cryoprotectants. The HIP complex may, in many cases, provide a simple alternative to obtaining a stabilized protein. A protein in the solid HIP complex has enhanced stability and resistance to degradation through storage, shipping, and handling. Chemical stability is conferred because the amount of water present is relatively low, as in lyophilized powders. To reconstitute the protein, the HIP complex is suspended in a diluent containing a significant chloride concentration (e.g., phosphate buffered saline (PBS) or normal saline). Most HIP complexes redissolve rapidly and completely, leaving a solution whose only additive is a small amount of SDS. The protein can also be stored as a stable entity by dissolving or suspending the HIP complex in an organic solvent or solvent mixture. To form an aqueous solution of the protein, the solution or suspension can be shaken with water containing chloride. In cases where the organic solvent is immiscible with water, the protein will partition into the water.

An additional embodiment of this invention is a method of incorporating proteins into lipid vesicles, liposomes, or detergent micelles. Shaking of an oil-water mixture with a HIP complex of a protein leads to emulsification, indicating that a HIP complex can more easily be introduced into emulsion delivery systems than the protein alone. Systems for such use can be designed using either the insoluble material in suspension formulations or in oil formulation, such as oil in water emulsions. Other examples include nasal and pulmonary aerosols, ophthalmic suspensions,

transdermal patches, lozenges, chewing gum, buccal and sublingual systems, suppositories.

Another aspect of this invention is the reduction of the bitter taste of drugs incorporated into HIP complexes, since only compounds in solution are tasted. Therefore, this invention includes a method for improving the taste of orally administered drugs by formation of insoluble HIP complexes with such drugs. The taste of a substance is detected by receptors in the tongue. A major approach to modifying the taste of a drug is to alter its solubility in saliva. If the solubility is sufficiently low the taste will not be noted. The low solubility of the HIP complex in biological fluids, including saliva, can be used to mask the flavor of a drug. Another way to mask taste is to partition the drug into an oil, such as olive oil. This can then be given as an oil in water emulsion with flavoring agents added to the outer water phase. HIP complex formation would provide the drug with the necessary high oil to water partition coefficient.

The term "hydrophobic ion-pairing (HIP)" as used in this disclosure refers to the interaction between anionic detergents containing long chain alkyl groups, such as SDS, and polypeptides. "HIP complex derivatives" are substances modified by formation of a hydrophobic ion-pair. The basis for this new technique is the interaction of an ionic detergent possessing some hydrophobic properties, and an oppositely charged polypeptide. This interaction has been termed HIP because it appears to be primarily electrostatic in nature.

As used in the present invention, the term "anionic detergents" encompasses any hydrophobic material that is a salt of an acid which can be employed to modify solubility properties in the described way, including sulfates, sulfonates,

phosphates, and carboxylates. Sulfates are the salts of the stronger acids in this series and, therefore, the most efficient at forming ion pairs. Provided that the alkyl chains or aryl rings are of 8 -18 carbons in length, they are potential candidates for HIP methodology. Presumably cationic detergents, such as dodecylamine hydrochloride or cetyltrimethylammonium bromide (CTAB), may also work for negatively charged peptides.

The methods used for measuring apparent partitioning coefficients are described in Example 1. The measurement of the behavior of the Gly-Phe-NH_2:SDS complex is described in Example 2. The behavior of the 8-Arg-vasopressin:SDS complex, leuprolide:SDS complex, neurotensin:SDS complex, and bradykinin:SDS complex are described in Example 3. The behavior of the insulin:SDS complex is described in Example 4. The dissolution of the insulin:SDS complex as a function of the organic solvent is described in Example 5. Further behavior of the leuprolide:SDS complex is described in Example 6. Example 7 describes the CD spectrum of the insulin:SDS complex. Example 8 describes the thermal stability of the insulin:SDS complex. Example 9 describes the behavior of other large proteins with SDS, specifically, human growth hormone. The behavior of bovine pancreatic trypstin inhibitor with SDS is described in Example 10, and Example 11 describes the behavior of human serum albumin with SDS. The melting point of the SDS:insulin HIP complex was studied (Example 12).

Example 13 describes a method for forming a fine HIP complex suspension suitable for pulmonary delivery. Example 14 describes a method for achieving uniform distribution of a protein throughout a hydrophobic polymer suitable for use as an injectable implant. Example 15 describes the use of the HIP complex for improved storage of proteins. The use of protein

precipitation in the HIP complex for protein purification is described in Example 16. A method of administering a protein dissolved as an HIP complex in organic solvent is described in Example 17. Example 18 describes the preparation of a drug with reduced bitter taste.

Example 1. Measurement of Apparent Partition Coefficients

The relative solubilities in two phases is given in terms of an apparent partition coefficient. The apparent partition coefficient is defined as the ratio of the equilibrium concentration in an organic phase to that in an aqueous phase. The actual value of the apparent partition coefficient, P, is dependent on the two solvent systems employed. In all cases herein described, the organic phase is 1-octanol and the aqueous phase is water alone or with a minimal amount of HCl added.

Apparent partition coefficients were measured by dissolving a peptide in 1.25 ml of an aqueous solution. Before SDS addition, the pH was measured on a Beckman pH meter. Upon addition of an SDS solution, the solutions turned cloudy and a precipitate formed immediately. An equal volume of 1-octanol was added and the mixtures agitated, and then left undisturbed for several hours. Prior to analysis, the tubes were spun for 10 minutes at 4000 g. Each layer was removed and the absorbance measured on a Beckman DU-64 UV-visible spectrophotometer using 1 cm quartz cells. All apparent partition coefficients were corrected for changes in pH with differing SDS concentrations.

Results are described as logarithms of the apparent partition coefficient. A log P value of 0 means that the compound is equally soluble in water and the organic phase. A positive log P value means the peptide is more soluble in the organic phase than in

water and a negative log P values indicate a greater aqueous solubility than in the organic solvent. All of the log P values reported herein have been corrected for slight changes in solubility with Ph.

Example 2. Apparent Partitioning Coefficient for Gly-Phe-NH_2.

The logarithm of the apparent water/1-octanol partition coefficients for Gly-Phe-NH_2 (Gly-Phe-amid, 0.6 mg/ml, pH about 5) and Gly-Phe (0.6 mg/ml at pH 7 and pH 3) as a function of SDS to peptide ratio are shown in Figure 1. Apparent partition coefficients were measured as described in Example 1.

In order for HIP to occur, the polypeptide must contain at least one basic group (either a lysine or arginine side chain or a free N-terminal amino group). Gly-Phe-NH_2 contains a single basic group, and at pH 7 forms a 1:1 complex with SDS. The complex precipitates from aqueous solution, but readily partitions into 1-octanol, as shown in Figure 1. For Gly-Phe itself, which exists in a zwitterionic form at neutral pH, a complex with SDS is formed with difficulty, and little enhancement of the partition coefficient is observed. However, by lowering the pH to less than 4, the carboxylate group of Gly-Phe becomes protonated, leaving the molecule with an overall positive charge and again, a hydrophobic ion pair can be formed. Partitioning of Gly-Phe at pH 3 mirrors the marked increase seen for Gly-Phe-NH_2. Therefore, even for acidic peptides, lowering the pH may permit hydrophobic ion pair complexes to be formed.

Example 3. Behavior of Protein:SDS complexes.

The logarithms of the apparent water/1-octanol partition coefficient for AVP (0.49 mg/ml, pH 5), leuprolide (LPA)(0.5 mg/ml, pH 6), neurotensin (NT)(0.y mg/ml, pH x), and bradykinin (BK)(0.y mg/ml, pH x) are

shown in Figure 2. Apparent partition coefficients were measured as described in Example 1.

Peptides larger than Gly-Phe-NH_2 can interact with SDS to form HIP-complexes with enhanced solubility in organic solvents. AVP is a nonapeptide hormone which controls water and salt elimination in the body. It contains two basic groups, the N-terminal amino group and the guanidinium side chain of Arg^8, and no acidic groups. Stoichiometric addition of SDS produces a precipitate from an aqueous solution (pH 7) which readily partitions into a 1-octanol (Figure 2). At a mole ratio of 2:1 (SDS:peptide), the solubility in 1-octanol actually exceeds the solubility in water by more than tenfold (i.e., log P > 1). Overall, the apparent partition coefficient for AVP was increased by nearly four orders of magnitude.

Example 4. Behavior of Insulin:SDS Complex.

The logarithm of the apparent partition coefficient of insulin as a function of SDS ratio is shown in Figure 3.

Polypeptides which contain both acidic and basic groups can also form hydrophobic ion pairs. Insulin contains six basic groups (one Arg, one Lys, two His, and two N-terminal amino groups) and four acidic groups. By lowering the pH to 2.5, all of the acidic groups (which are carboxylic acids) become protonated and the only remaining charges are due to the basic functional groups, producing an overall charge of +6.

The solubility of insulin is altered dramatically upon addition of stoichiometric amounts of SDS (Figure 3). The solubility of an insulin-SDS complex approaches 1 mg/ml (0.17 mM) in 1-octanol, and its apparent partition coefficient increases by nearly four orders of magnitude. At higher SDS concentrations, the apparent partition coefficient decreases, because the solubility of insulin in water increases again,

presumably due to micelle formation.

Example 5. Dissolution of Insulin-SDS Complex as a Function of the Organic Solvent.

Dissolution of insulin-SDS complexes in other solvents was investigated as well (Table I). Precipitates of SDS-insulin complexes were isolated and added to various organic solvents. Some degree of polarity appears to be necessary to obtain measurable solubility in the organic phase, as partitioning into chlorocarbons (CH_2Cl_2, 1-chlorooctane, and CCl_4) and alkanes (mineral oil, hexane) could not be detected using UV-visible absorption spectroscopy. Besides alcohols, SDS-insulin complexes are soluble in N-methylpyrollidinone (NMP), trimethylphosphate (TMP), polyethylene glycol, ethanol, and t-butanol.

TABLE 1. PARTITIONING OF INSULIN INTO NONAQUEOUS SOLVENTS

Organic Solvent	Log P	Apparent Sol. (mg/ml)
1-octanol	≥ 1.2	≥ 1.0
CCl_4	not detectable	insoluble
Mineral Oil	not detectable	insoluble
CH_2Cl_2	not detectable	insoluble
Dimethoxyethane	not detectable	not determined
Hexane	not detectable	insoluble
1-Chlorooctane	not detectable	insoluble
THF	miscible	not determined
Acetone	miscible	not determined
Ether	not detectable	insoluble
DMF	not determined	≥ 1.0
NMP	miscible	≥ 1.0
Ethyl acetate	miscible	insoluble
PEG 400	miscible	≥ 0.2
Trimethyl phosphate	miscible	≥ 0.15
Ethanol	miscible	≥ 1.0
i-Propanol	miscible	≥ 1.0
Methanol	miscible	≥ 1.0
Propylene Glycol	miscible	≥ 0.5
TMP	miscible	≥ 0.2
Trifluoroethanol	miscible	≥ 1.5

Example 6. Behavior of Leuprolide:SDS Complex

Leuprolide acetate is a luteinizing hormone releasing hormone (LHRH) agonist used in the treatment of endometriosis. It contains 9 amino acid residues and two basic functionalities (a histidine and an arginine group). Both termini are blocked. Stoichiometric amounts of SDS were added to an aqueous solution of leuprolide (0 and 0.5 mg/ml, pH 6.0), resulting in formation of a precipitate. The apparent

partition coefficient of the SDS-leuprolide complex (Figure 2) exhibited a log P into 1-octanol greater than 1.0.

Example 7. CD Spectrometry of the SDS-Insulin Complex.

Two important considerations for proteins dissolved in nonaqueous solvents are whether native structures are retained and whether the material can be extracted back into an aqueous phase. The secondary composition of a 6:1 SDS-insulin complex dissolved in neat 1-octanol at 5° C is shown in Figure 3. The insulin concentration was 61 ug/ml.

CD spectra were recorded on an Aviv 62DS spectrophotometer equipped with a thermoelectric temperature unit. All temperatures were measured ± 0.2° C. Samples were placed in strain-free quartz cells (pathlength of 1 mm) and spectra obtained taking data every 0.25 nm using a three second averaging time, and having a spectral bandwidth of 1 nm.

Analysis of the CD spectrum, using an algorithm based on the methods of Johnson (1990) Genetics 7:205-214 and van Stokkum et al. (1990) Anal. Biochem. 191:110-118, indicates that the alpha-helix content of insulin in octanol is 57%, similar to that found for insulin in aqueous solution (57%) (Melberg and Johnson (1990) Genetics 8:280-286) and in the solid state by x-ray crystallography (53%) (Baker et al. (1988) Phil. Trans. R. Soc. London B319, 369-456). The spectra are slightly more intense than those reported for insulin in water (Pocker and Biswas (1980) Biochemistry 19:5043-5049; Melberg and Johnson (1990) supra; Brems et al. (1990) Biochemistry 29:9289-9293). The relative intensity of the 222 nm band to the 208 nm band is similar to that observed for insulin at high concentrations (Pocker and Biswas (1980) supra). This represent the first example of native-like structure in

a protein dissolved in a neat organic solvent.

Figure 4 shows the far ultraviolet CD spectrum of insulin extracted from 1-octanol into an aqueous solution of 0.10 M HCl. The pathlength was 1 mm, the sample concentration 53 ug/ml, and the sample temperature 5° C. Upon shaking an octanol solution of insulin with an aqueous solution containing 0.10 M HCl, insulin can be extracted back into the aqueous phase, presumably due to replacement of the SDS counterion with chloride. Lower HCl concentrations did not affect extraction of insulin from 1-octanol. Examination of the CD spectrum of the redissolved material (Figure 4) indicates an overall structure similar to that of native insulin.

Example 8. Increased Thermal Stability of the SDS:insulin Complex.

The stability of insulin to thermal denaturation is difficult to assess as chemical degradation rates are rapid at elevated temperatures (Ettinger and Timasheff (1971) Biochemistry 10:824-831). In aqueous solution, the thermal denaturation of insulin occurs at a T_m of about 65° C [define T_m]. The T_m of insulin in 1-octanol has been measured, following molar ellipticity at 222 nm, to occur at 98° C (Figure 6), which is more than 30 degrees above that observed in water. This observation supports the conclusion that proteins dissolved in organic solvents demonstrate exceptional thermal stability. Although prior reports have observed that proteins suspended in organic solvents exhibit increased chemical stability due to lack of water (Ahern and Klibanov (1987) references), the present disclosure is the first report to find increased protein stability of the SDS:protein complex in organic solvent with respect to denaturation. Furthermore, as shown in Figure 9, the SDS-insulin complex appears to maintain its native structure in 1-

octanol, even after prolonged heating at 70° C for more than 1 hour.

Example 9. Behavior of Large Proteins Complexed with SDS.

Larger proteins can also form complexes with SDS. At pH 7.8, the aqueous solubility of human growth hormone (hGH) was not affected by addition of SDS, even at ratios of 100:1. However, at pH 2, hGH precipitates from aqueous solution at SDS ratios ranging from 10:1 to 40:1. At higher SDS concentrations, hGH redissolves, presumably via micellar solubilization. The hGH precipitate was not found to be soluble in 1-octanol, as determined by spectrophotometric assay. However, it was easily suspended in water and various oils, such as olive oil.

Example 10. Behavior of Bovine Pancreatic Trypsin Inhibitor Complexed with SDS.

Other proteins can also form complexes with SDS. Bovine pancreatic trypsin inhibitor (BPTI) is a small basic protein (MW 5900) with a well defined and stable structure (Wlodawer et al. (1984) J. Mol. Biol. 180:301-329, and (1987) J. Mol. Biol. 193:145-156). At pH 4, it partitions into 1-octanol upon addition of SDS (Figure 7). As with insulin, the structure is maintained (data and shown) and the SDS-BPTI complex is soluble in other solvents as well, such as NMP and trimethyl phosphate (TMP). In TMP, the globular structure is compromised, as determined by CD spectroscopy. Apparently, TMP is a strong enough solvent to displace water from the hydration sphere and destabilize the structure of BPTI. This mechanism of protein denaturation has been described in detail by Arakawa and Timasheff (1982) Biochemistry 21:6536-6544, and (1982) Biochemistry 21:6545-6552.

Example 11. Behavior of HIP Complex Formation with Human Serum Albumin.

Stoichiometric addition of SDS to human serum albumin (HSA) (MW 68 kD) produces precipitates as a hydrophobic ion pair complex is formed. While partitioning into 1-octanol could not be detected by UV- visible absorption spectroscopy, the SDS-HSA complex was found to be soluble in NMP (Figure 8), yielding solutions of concentrations greater than 1 mg/ml (pathlength = 1 cm, sample temperature = 27°C). Without SDS, the solubility of HSA in NMP is less than 0.03 mg/ml.

Example 12. Melting Point of SDS:insulin complex.

The melting point (MP) of SDS:insulin ion pairs in 1-octanol was studied at SDS:insulin rations ranging from 1:1 to 1:24.

Insulin at 1 mg/ml in 0.005 N HCl was prepared containing SDS at 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 15, 18, 21 and 24 moles of SDS per mole of insulin. Equal volumes of octanol were added to each SDS:insulin solution to partition the insulin into the octanol phase. The concentration of the SDS:insulin complex extracted into the octanol was estimated by its absorbance at 278 nm and the solution diluted to 200 ug/ml. The melting point of the various insulin in octanol solutions was then determined with an AVIV 62DS circular dichroism spectrometer. Both circular dichroism (CD) signal and light scattering (as measured by changes in absorbance) were measured at 222 nm and the melting point determined by an inflection point in the measured scan.

Figure 9 shows the graph of melting point as a function of SDS:insulin molar ratios, with an apparent maximum at 6:1 molar ratio and a melting point of about 116°C. The molar ratio of 6:1 is also the stoichiometric ratio and show the highest thermal

stability for insulin in octanol.

Figure 10 shows a typical CD scan at 222 nm as a function of temperature. A melting point of 106°C was determined by the maxima of the first derivative of the pictured data. Figure 11 shows a typical absorbance scan at 222 nm as a function of temperature and effectively mimics the CD scan, showing a melting point of 106°C.

Example 13. Formation of a Fine Suspension HIP Complex for Pulmonary Delivery.

For the formation of particles for pulmonary delivery, a protein solution is stirred vigorously using a homogenizer. SDS is added dropwise to the agitated solution. Particles in the 2-10 micron range are obtained. These particles are separated from the mixture by centrifugation or filtration. The particles are then suspended in a mixture of Freon® 11 and 12, such than when placed in a meter dose inhaler, a therapeutic amount of protein is delivered on each actuation.

Example 14. Uniform Distribution of Protein Throughout a Hydrophobic Polymer for Use an an Injectable Implant.

The biodegradable polymer consisting of a 50:50 mixture of poly-lactic acid and poly-glycolic acid is dissolved in a volatile organic solvent, such as N-methyl-pyrrolidone (NMP). An appropriate amount of an HIP-protein complex such as insulin-SDS (0.5%-5.0% by weight relative to the polymer) is dissolved in the same solvent. The two solutions are mixed and stirred for one hour. After the mixing is complete, the solvent is removed by evaporation. This is done in a mold to form an implant, or by a spray drying procedure to form small uniform particles for injection. The resulting solid material can also be ground to a powder and formulated as an injectable suspension. The

protein is released from these systems as the polymer biodegrades and the HIP complex hydrolyses.

Example 15. Use of HIP Complex Formation for Protein Storage.

The HIP complex is formed by dissolving the protein or polypeptide in water at minimal ionic strength. The pH is adjusted to as low a pH value as is practical to ensure stability and activity. A stock solution of SDS is added so that the number of equivalents of SDS matches the number of basic groups. For insulin, the pH is adjusted to 2.5, and 6 molar equivalents of SDS are used per mole of insulin. The resulting complex precipitates from solution, is collected, and dried at room temperature. The solid HIP complex may be stored at higher humidities and temperatures than the native proteins without noticeable loss of activity.

Dissolution in a non-reactive organic solvent, such as 1-octanol, produces a true solution of a protein. The HIP complex of insulin stored in 1-octanol is much more stable than insulin in water, as shown by its enhanced thermal stability.

Example 16. Use of HIP Complex Formation for Protein Purification.

The hydrophobicities of HIP complexes of proteins will differ according to the fraction of the protein's surface covered by the alkyl sulfate molecules. In turn, the HIP protein complexes are separated using a variety of methods, including hydrophobic interaction columns.

Further, proteins may be purified by selective precipitation out of solution. For example, a protein is separated from additives such as human serum albumin (HSA), which may be present in amounts 20-50 times greater than the protein. Since HSA does not

precipitate out of solution at pH 5.0 with SDS, a basic protein may be selectively precipitated and purified from HSA under those conditions.

Example 17. Use of HIP Complex Dissolved in an Organic Solution for Administration of a Protein To a Patient.

The administration of HIP complexes to a patient may be accomplished in a number of ways. A biodegradable polymer/HIP complex system may be dissolved in an organic solvent, for example N-methyl pyrrolidone, and injected subcutaneously to form an implant, processed to form microspheres which can be injected subcutaneously or intramuscularly, processed to form an implant which is placed surgically under the skin, or given orally as part of an oral delivery system for peptides and proteins. The solid HIP complex may also be prepared as a suspension or a nonaqueous solution, which may be injected or placed on the skin where the complex may partition into the skin. The HIP complex may also be nebulized and administered to a patient via inhalation, for pulmonary drug delivery. The HIP complex may also be formulated to be given orally, such that it is protected from degradation in the stomach via an enterically coated capsule, and released in either the upper or lower intestinal tract. The HIP complex may be loaded alone or in conjunction with oils, bile salts, or other enhancers to increase absorption. The HIP complex may also be suspended or dissolved in oil and introduced to the patient as a rectal or vaginal suppository.

Example 18. Preparation of a Drug With Reduced Bitter Taste.

The low solubility of the HIP complex results in diminished taste of bitter tasting drugs taken orally. The HIP complex may also be dissolved in oil so as to further reduce bitter taste. The slow rate of

hydrolysis, especially in an oil-type vehicle, prevents the bitter tasting drug from dissolving in the mouth and being tasted.

CLAIMS

1. A method for forming a homogeneous solution of an organic molecule comprising:
a) adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate;
b) isolating said precipitate;
c) dissolving said precipitate in an organic solvent to form a homogeneous solution.

2. The method of claim 1 wherein said organic molecule is a peptide or protein containing at least one basic group.

3. The method of claim 1 wherein said anionic detergent is sodium dodecyl sulfate.

4. The method of claim 1 wherein said organic solvent is 1-octanol.

5. The method of claim 1 wherein said organic molecule has an improved thermal stability in said homogeneous solution over the uncomplexed molecule.

6. An method of forming an injectable implant comprising:
a) adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate;
b) isolating said precipitate;
c) co-dissolving said precipitate and a hydrophobic polymer in an organic solvent to form a homogeneous distribution of the organic molecule within said polymer.

7. A method of uniformly distributing an organic molecule in a hydrophobic polymer comprising:
a) adding a sufficient amount of an anionic detergent

to an organic molecule to form a precipitate;
b) isolating said precipitate;
c) co-dissolving said precipitate and a hydrophobic polymer in an organic solvent to form a homogeneous distribution of the organic molecule within said polymer.

8. A method of controlling the size of particle formation of a drug comprising controlling the rates of the mixing of a protein solution and the addition of an anionic detergent to the protein solution.

9. The method of claim 6 wherein a particle size of 2-10 microns is produced by rapid agitation of a protein solution with dropwise addition of an anionic detergent.

10. A method of producing a protein suitable for pulmonary delivery comprising:
a) rapidly mixing a protein solution;
b) adding an anionic detergent dropwise to said rapidly mixing protein solution such that the protein forms a particle size of between 2 - 10 microns;
c) isolating said particles from said solution; and
d) suspending said precipitate in a suitable pharmaceutical carrier.

11. A method for incorporating proteins into lipid vesicles comprising:
a) adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate;
b) isolating said precipitate; and
c) dissolving said precipitate in an oil.

12. A method for reducing the bitter taste of drugs comprising administering a bitter tasting drug in an HIP complex by:

a) adding a sufficient amount of anionic detergent to a bitter-tasting drug in solution wherein an insoluble precipitate is formed;
b) isolating said precipitate;
c) administering said precipitate orally to a patient in need of the drug thereof.

13. A method for enhancing the thermal stability of a protein comprising:
a) adding a sufficient amount of an anionic detergent to a protein to form a precipitate;
b) isolating said precipitate;
c) dissolving said precipitate in an organic solvent.

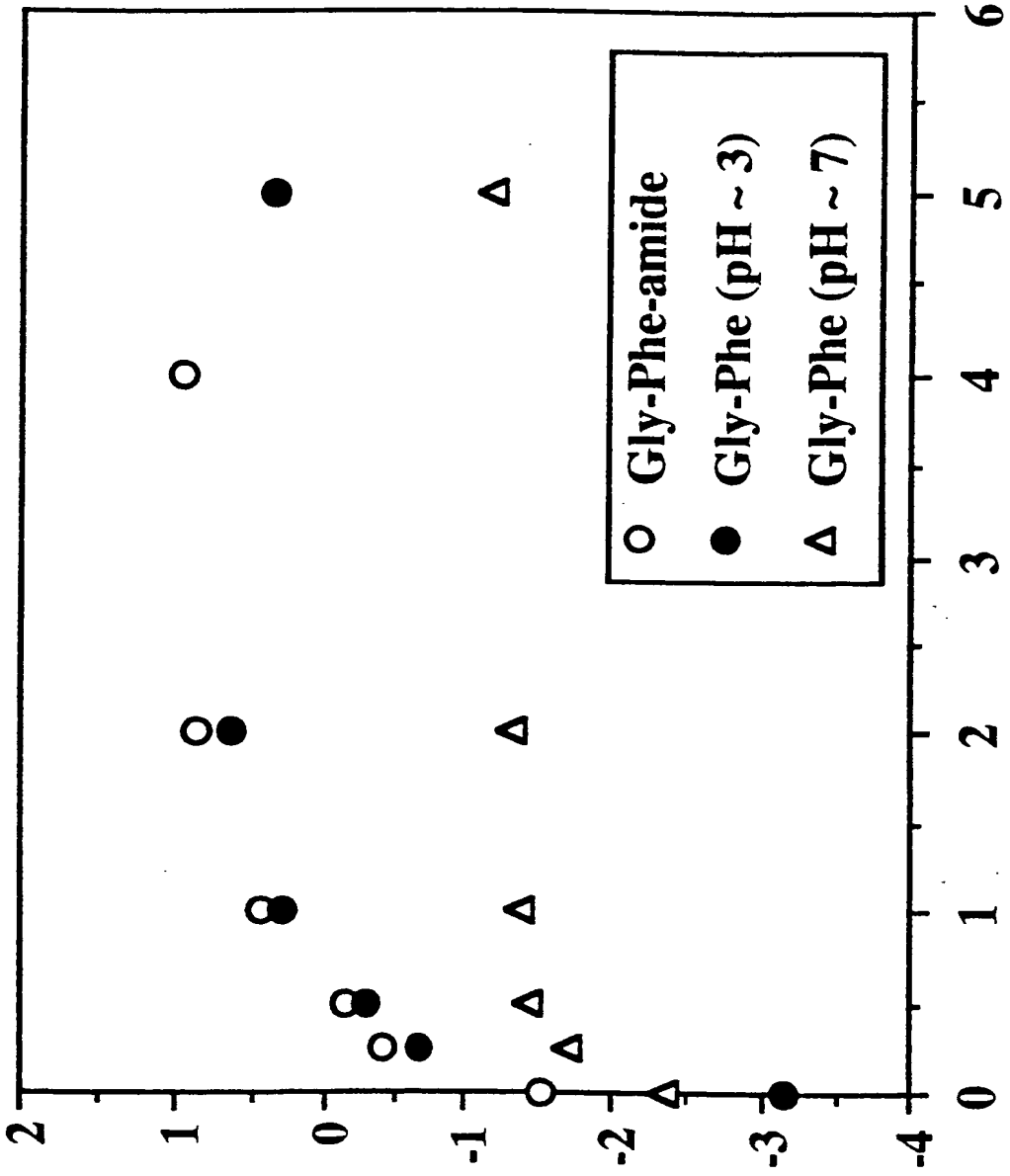
14. A method for producing an enzymatically active protein in an organic solvent comprising:
a) adding a sufficient amount of an anionic detergent to a protein to form a precipitate;
b) isolating said precipitate;
c) dissolving said precipitate in an organic solvent.

15. A method of controlling the release of a protein into an aqueous solution comprising forming HIP complexes of specific sizes by controlling the rates of the mixing of a protein solution and the addition of an anionic detergent to the protein solution.

16. An improved method for the long-term storage of organic molecules comprising:
a) adding a sufficient amount of an anionic detergent to an organic molecule to form a precipitate; and
b) isolating said precipitate.

17. The method of claim 16 wherein said organic molecule is a protein.

Figure 1



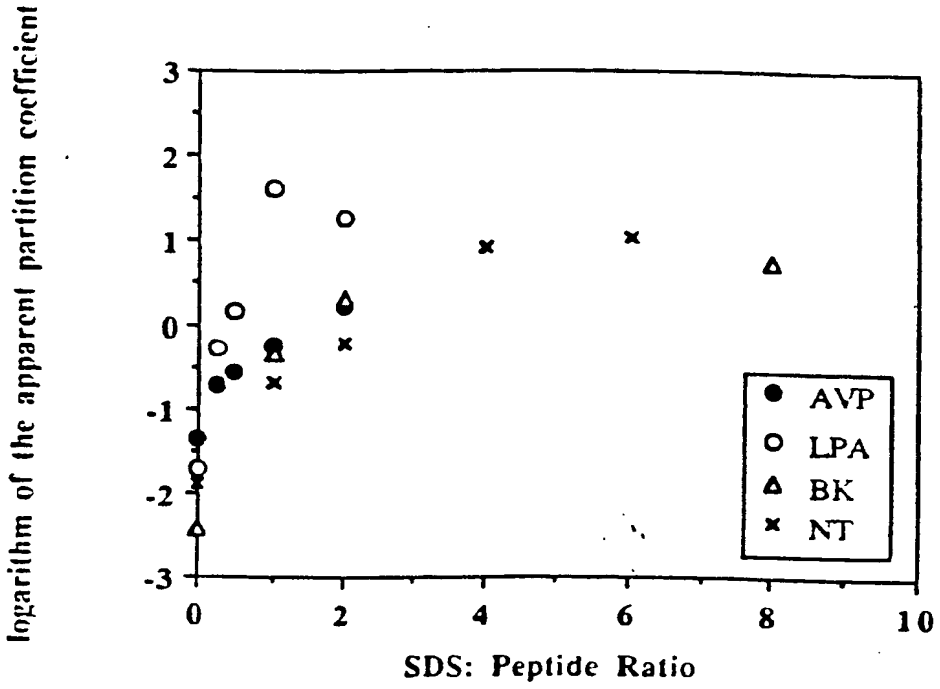


FIG. 2. The logarithm of the apparent partition coefficient (relative solubility in water and 1-octanol) of AVP (O, 0.5 mg/ml, pH 7), leuprolide (●, LPA, 0.5 mg/ml, pH 6), neurotensin (X, NT, 0.y mg/ml, pH x), and bradykinin (Δ, BK, 0.y mg/ml, pH x).

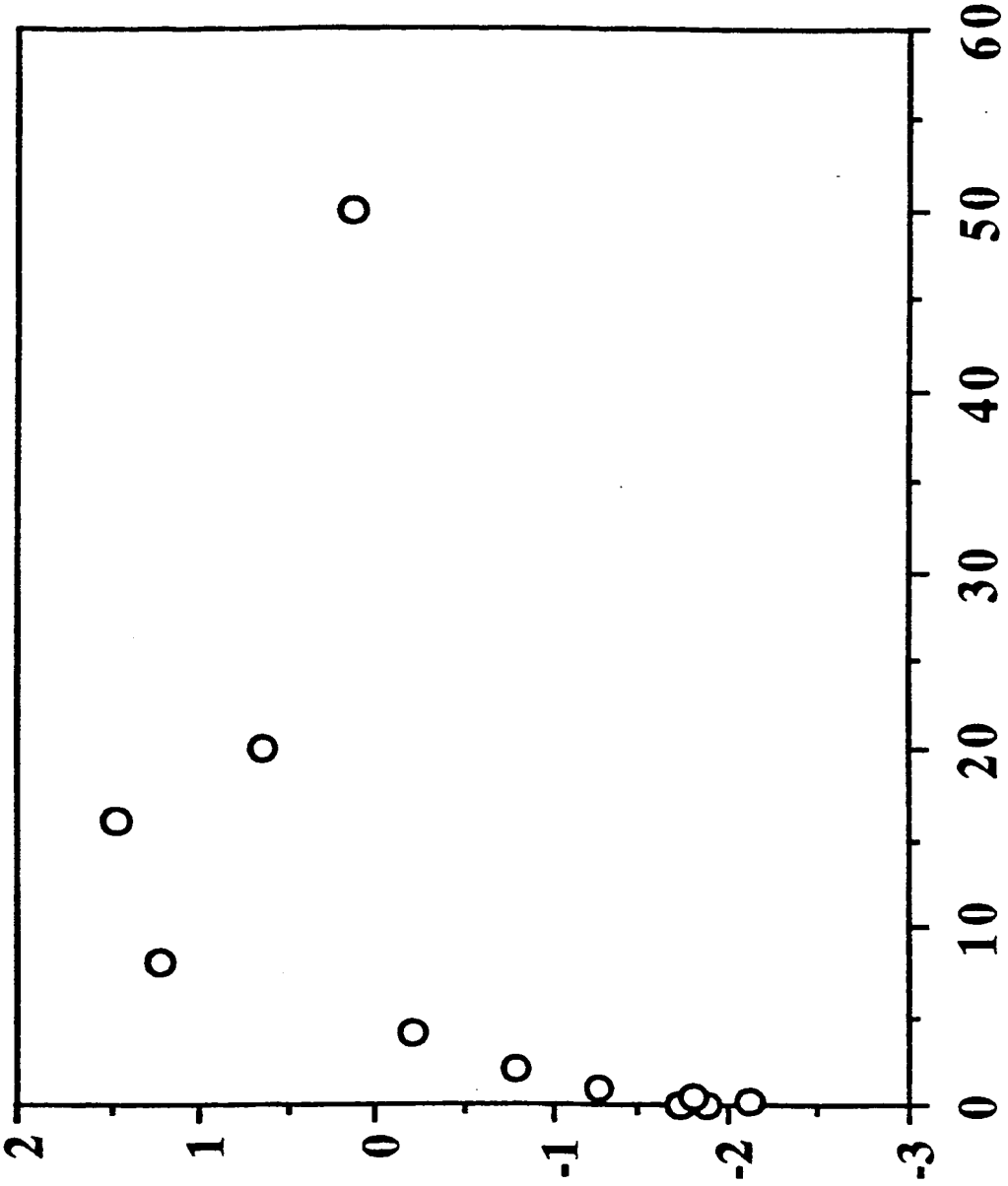


Figure 3

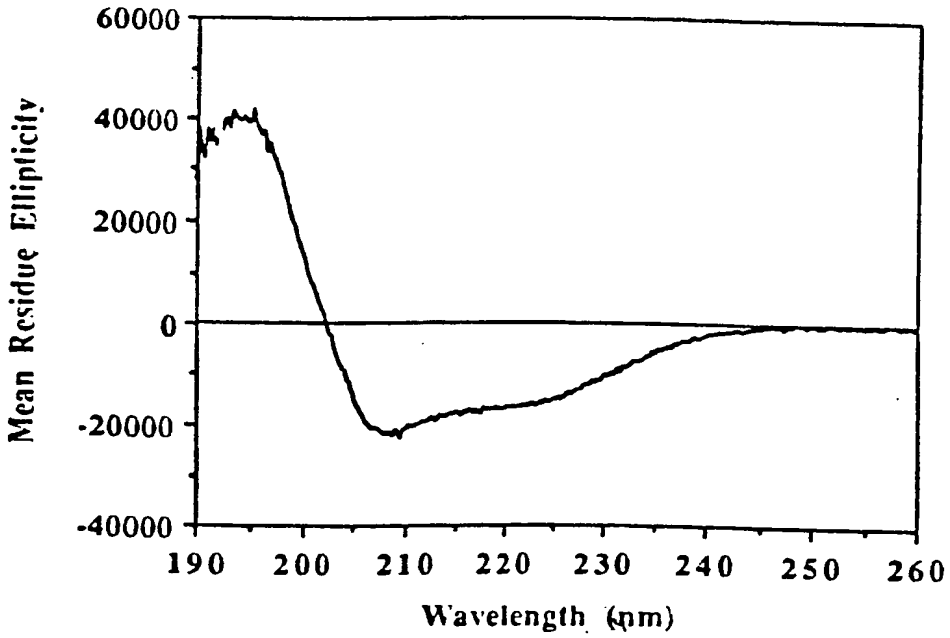


FIG. 4. Far ultraviolet CD spectra of a 6:1 SDS-insulin complex in neat 1-octanol. The pathlength was 1mm. The insulin concentration was 61 µg/ml for the sample at 5° C.

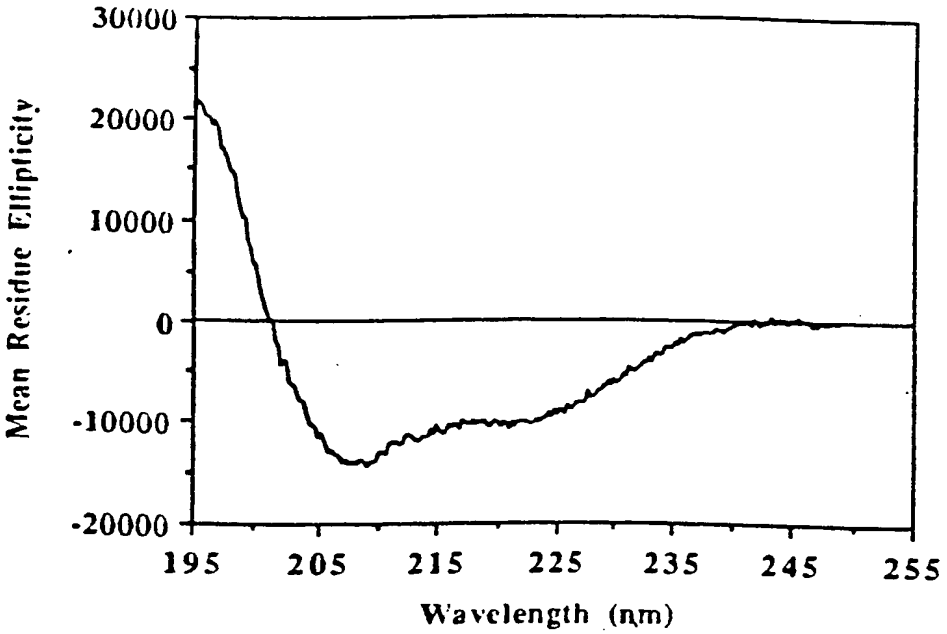


FIG. 5. Far ultraviolet CD spectra of insulin extracted from 1-octanol using an aqueous solution of 0.10 M HCI. The pathlength was 1 mm, the sample concentration was 53 µg/ml, and the sample temperature was 5° C.

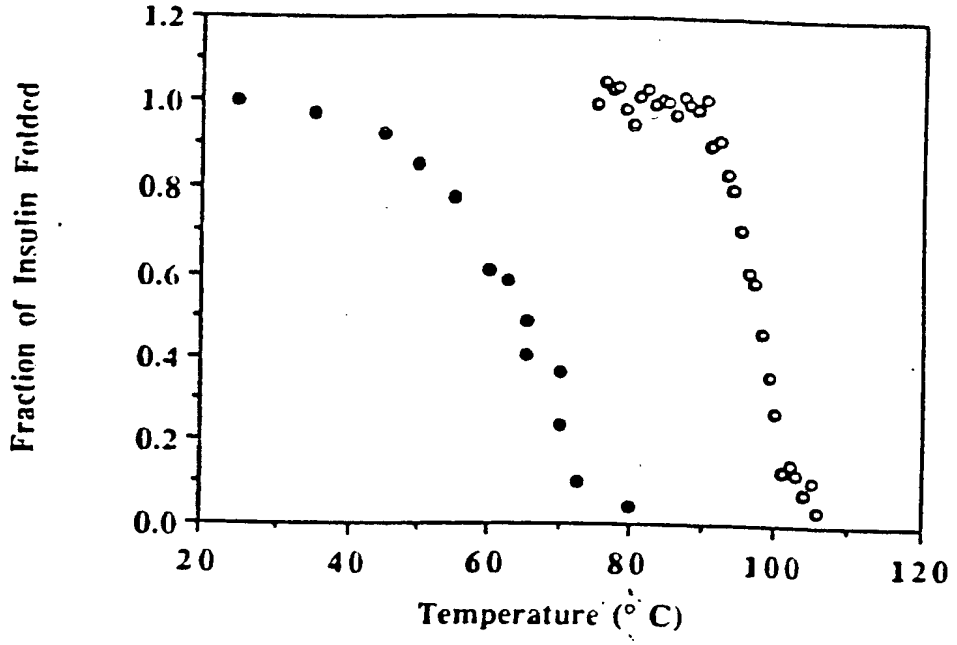


FIG. 6. The effect of temperature on the denaturation of insulin dissolved in water (●) and 1-octanol (O). The fraction folded was determined by monitoring the molar ellipticity at 222 nm.

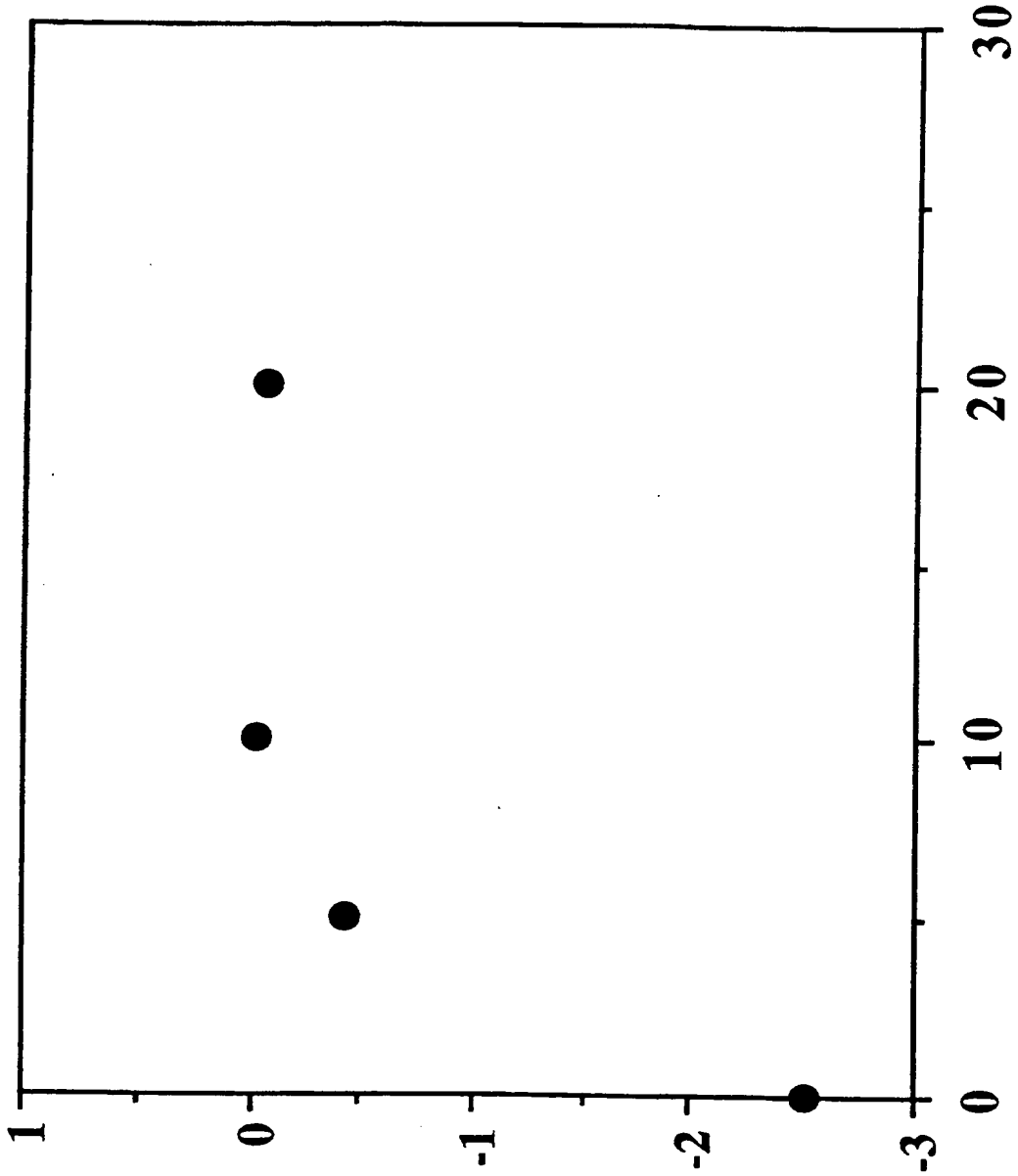


Figure 7

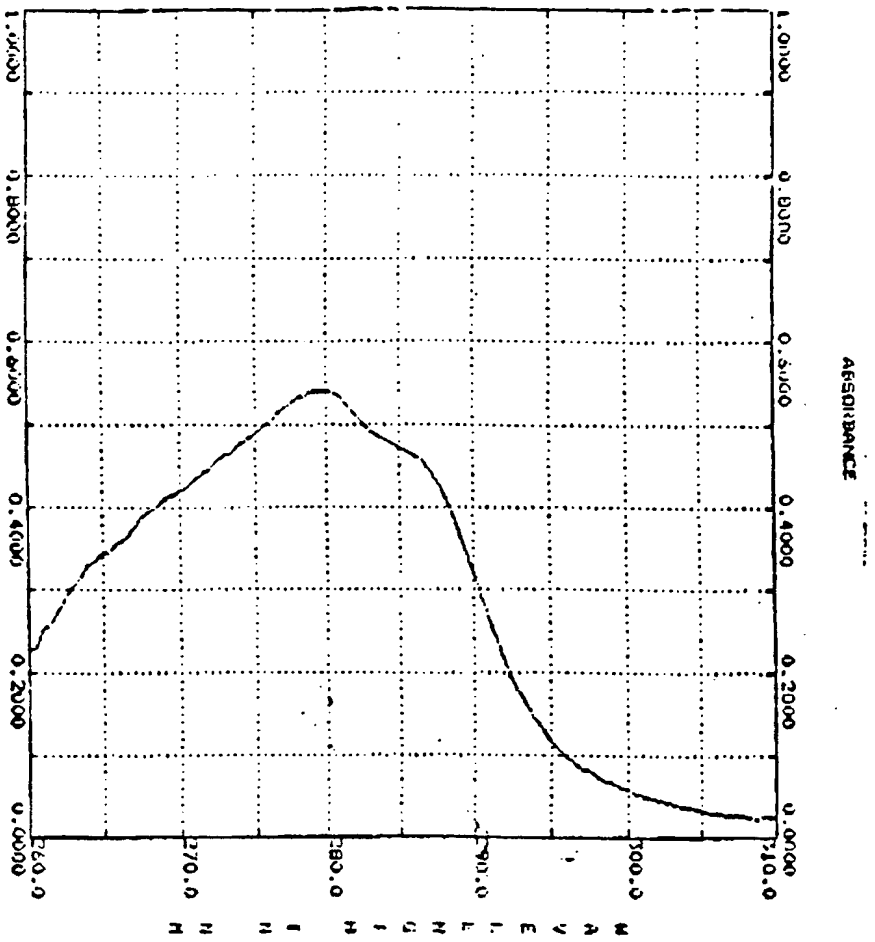


FIG. 8 UV-visible absorption spectrum of HSA in NMP (50:1 SDS to HSA ratio). T pathlength was 1 cm and the sample temperature was 27° C.

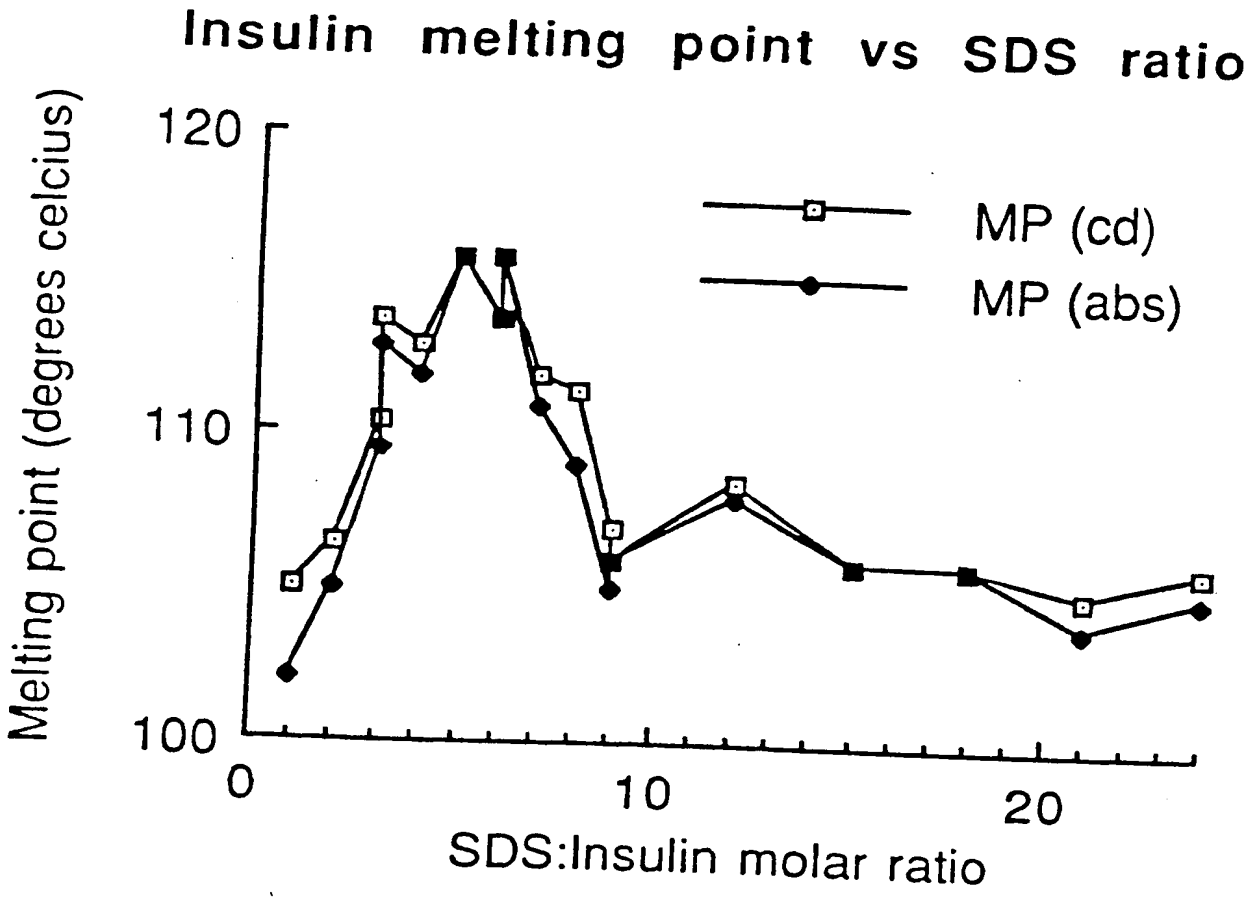


FIG. 9. Plot of melting point as a function of SDS:Insulin ratio.

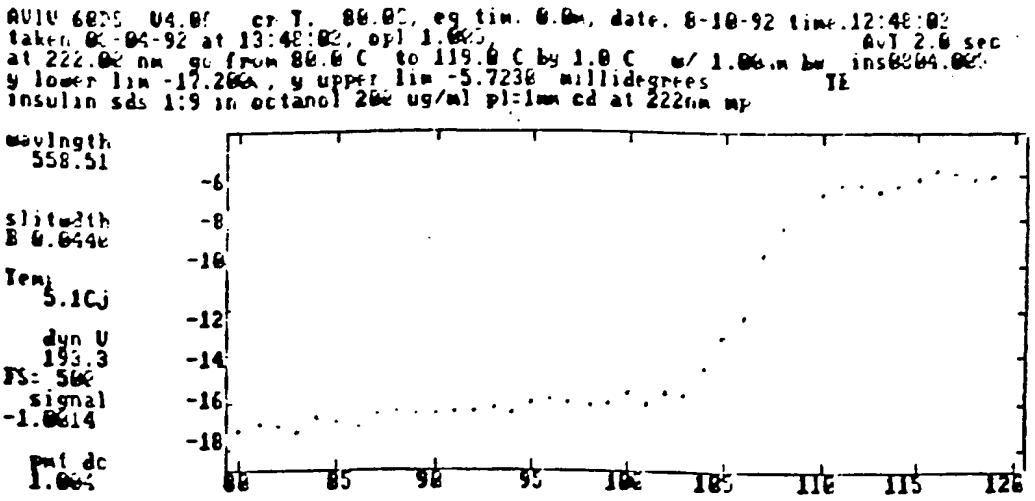


FIG. 10. CD scan at 222nm for the 9:1 SDS:I ratio in octanol.

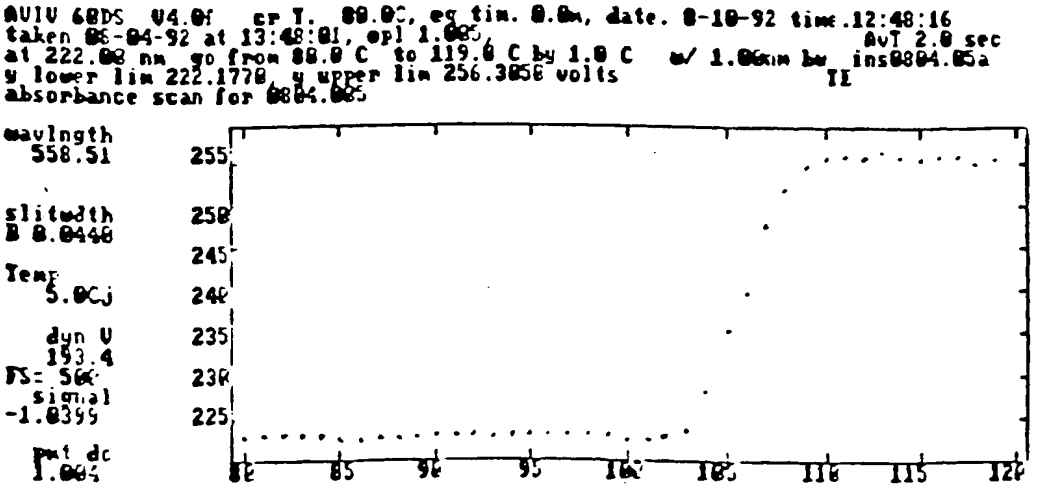


FIG. 11. Absorbance scan at 222nm for the 9:1 SDS:I ratio in octanol.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09576

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00
US CL :514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 424/418, 424/428, 530/418, 530/419, 530/422, 530/424.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
peptide, solvent or alcohol, solubility, anionic detergent, SDS.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,654,132 (Takagi et al.) 31 March 1987, col 1, lines 28-44.	1-5, 13-14, 16-17
A	US, A, 4,992,271 (Fernandes et al.) 12 Feb 1991, col 4, lines 1-12.	1-5, 13-14, 16-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
08 December 1993

Date of mailing of the international search report
JAN 05 1994

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Authorized officer
DAVID LUKTON

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09576

Box I Observations where certain claims wer f und unsearchable (Continuation of item 1 f first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 13-14, 16-17.

Remark n Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09576

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-5, 13-14, 16-17 drawn to a method for forming a homogeneous solution of an organic molecule, or for the long-term storage of organic molecules, classified in, e.g. Class 514/2.

II. Claims 6-7, drawn to a method of forming an injectable implant, classified in e.g., Class 424/423.

III. Claims 8-10, drawn to a method of controlling the size of particle formation of a drug, or producing a protein suitable for pulmonary delivery, classified in e.g., Class 424/418.

IV. Claim 11, drawn to a method for incorporating proteins into lipid vesicles, classified in, e.g., Class 424/450.

V. Claim 12, drawn to a method for reducing the bitter taste of drugs, classified in, e.g., Class 424/410.

VI. Claim 15, drawn to a method of controlling the release of a protein into an aqueous solution, classified in, e.g., Class 424/489.